Inhibition of phosphodiesterase 4 amplifies cytokine-dependent induction of arginase in macrophages

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Arginase has been implicated in the pathogenesis of asthma in several ways: first, by reducing arginine availability it can limit NO production; second, the ornithine produced by arginase can be converted to polyamines and/or proline, thus providing support for cell proliferation and collagen synthesis, respectively, both of which are important processes in airway remodeling in asthma and other pulmonary diseases (15). Several cytokines involved in asthma, including interleukin-4 (IL-4) and transforming growth factor (TGF)-β, are potent inducers of arginase activity (2, 5, 13, 23). Adenosine 3',5'-cyclic monophosphate (cAMP), whose intracellular levels can be increased in response to various inflammatory stimuli, also is an inducer of arginase (22, 25).

Considerable interest exists in using phosphodiesterase 4 (PDE4) inhibitors for chronic inflammation since PDE4 is the primary PDE in inflammatory cells (3). The phosphodiesterases, consisting of 11 families, are enzymes that hydrolyze the cyclic nucleotides cAMP and/or cGMP, depending on the specific PDE (3). As elevation of cAMP levels can result in both vasodilation and reduced inflammation in inflammatory pulmonary diseases, there is interest in potential clinical applications of inhibitors of PDE4, which specifically hydrolyze cAMP. Since both disease-related cytokines and treatment with PDE inhibitors could result in increased arginase, the following study tested the hypothesis that PDE4 inhibition would amplify arginase expression by various cytokines. This hypothesis was tested with the RAW 264.7 murine macrophage cell line, and similar studies were performed using human alveolar macrophages (AM) obtained by BAL.

METHODS

Reagents. Rolipram, 3-isobutyl-1-methylxanthine (IBMX), and forskolin were obtained from Sigma, and 8-bromo-cAMP was from Roche Applied Science. Recombinant human TGF-β, recombinant human IL-4, and recombinant murine IL-4 were purchased from R&D Systems. Dulbecco’s modified Eagle’s medium (DMEM) was pur-
chased from BioWhittaker. RPMI 1640 was purchased from GIBCO. American Radiolabeled Chemicals was the supplier for [14C-guanido]-L-arginine.

Cell culture. The RAW 264.7 murine macrophage cell line was obtained from American Type Culture Collection and maintained in DMEM containing 10 mM HEPES (pH 7.5), 2 mM glutamine, penicillin (200 U/ml), streptomycin (200 μg/ml), and 10% fetal calf serum (low endotoxin, HyClone Laboratories). On the day of the experiment, the medium was changed and cells were treated with IL-4 (5 ng/ml) or TGF-β (10 ng/ml), with or without the PDE4 inhibitor rolipram (10 μM; added 30 min before addition of cytokines). After cytokine stimulation, cells were harvested at 6 and 20 h for determination of mRNA levels or at 20 h for measurements of arginase activity and immunoblotting.

Determination of intracellular cAMP. RAW 264.7 cells were incubated in the presence or absence of 10 μM rolipram for 45 min. Cells were washed once with ice-cold PBS and then extracted with 0.1 N HCl. Insolubles were removed by centrifugation, and the supernatant was used for determinations of cAMP concentrations with a cAMP Enzyme Immunoassay kit (Cayman Chemical) and for measurement of protein concentration with the Bio-Rad Protein Assay Kit, according to the manufacturer’s instructions.

Arginase activity. Arginase activity in cell lysates was determined as the conversion of [14C-guanido]-L-arginine to [14C]urea, which was then converted to 14CO2 by urease, and trapped as Na214CO3, which was counted on a scintillation counter as described previously (22). Protein concentrations were determined by the bicinchoninic acid (BCA) protein assay kit from Pierce and used to calculate arginase-specific activities of cell lysates.

Isolation and analysis of RNA. Total cellular RNA was isolated as described (22) with TRIzol reagent (Invitrogen) and treated with SuperScript II Reverse Transcriptase (Invitrogen) with random primers (Promega) in a total volume of 30 μl. Control reactions without reverse transcriptase were performed to confirm the absence of DNA contamination. Two-step quantitative real-time PCR was conducted using Applied Biosystems SYBR GREEN PCR master mix on an ABI Prism 7700 sequence detection system. PCR primers for murine arginase I, arginase II, and hypoxanthine-xanthine phosphoribosyltransferase (HPRT) are shown in Table 1. The abundance of HPRT mRNA was measured as an internal reference. All samples were run in duplicate. Control experiments were performed to establish the specificity and efficiency of primers for all mRNA species: Representative melting curve analyses for arginase I, arginase II, and HPRT amplification products indicated one specific product for each and no primer-dimer formation. Amplification plots demonstrated that the abundance of HPRT mRNA was invariant throughout all treatments, thus validating its use as an internal control. PCR products for each mRNA were analyzed by agarose gel electrophoresis to confirm that a single product of the predicted length was produced.

Quantification of relative gene expression was done using the comparative threshold cycle (Ct) method (12). Validation methods were determined over a 60-fold range to determine equal primer efficiency. Ct values were averaged for duplicate wells and subtracted from corresponding Ct of the internal reference RNA to obtain ΔCt values. The averaged control ΔCt was subtracted from the experimental ΔCt to yield ΔΔCt. The fold increase was calculated as 2−ΔΔCt for experimental vs. control (12).

Western blot. We immunoblotted 20 μg of the same lysates used for arginase activity determinations for arginase I as previously described (22). To detect arginase I, we used a primary anti-arginase I IgY [1:50,000 in 1% bovine serum albumin-1% nonfat milk (BSA/NFM)] in Tris-buffered saline containing 0.1% Tween 20 (TBS-T; 4°C overnight) and a secondary rabbit, anti-chicken IgY-horseradish peroxidase conjugate (1:5,000 in 1% BSA/1% NFM in TBS-T; Jackson ImmunoResearch Laboratories). Equal loading of protein was verified with Sypro Ruby Protein Blot Stain (Invitrogen).

Analysis of arginase I transcription. RAW 264.7 cells were transfected with a luciferase reporter gene driven by a 4.78-kb murine arginase I promoter as previously described (7) and selected with antibiotic G418 (1.0 mg/ml) to obtain stable transfectants. Stably transfected cells were maintained in DMEM containing 10 mM HEPES (pH 7.5), penicillin (200 U/ml), streptomycin (200 μg/ml), 10% fetal calf serum, and G418 (0.5 mg/ml, removed from media when cells were plated for experiment). Cells were stimulated with IL-4 (5 ng/ml) or TGF-β (10 ng/ml) for 8 h with or without rolipram (10 μM). Cells were lysed in Passive Lysis Buffer (Promega), and luciferase activity was assayed by the Luciferase Assay System (Promega) according to the manufacturer’s instructions. Protein concentrations were determined by BCA protein assay kit from Pierce and used to calculate luciferase-specific activities of cell lysates.

Patient summary. BAL fluid was obtained from healthy volunteers enrolled as controls (ages 23–54 yr) in an ongoing asthma study. All procedures were approved by the University of Pittsburgh Institutional Review Board, and all test subjects had signed a statement of informed consent. All subjects had a normal spirometry and no history of asthma or allergic disease. Skin test responses to common allergens were performed, and a positive result to an allergen resulted in exclusion.

Human AM. AM were obtained by BAL as previously described (4), counted, and plated. After 2 h, cells were washed with fresh RPMI 1640 containing 10 mM HEPES (pH 7.5), penicillin (200 U/ml), streptomycin (200 μg/ml), 2 mM L-glutamine, and 5% human serum (from a pooled stock from control patients) to remove unattached cells, leaving an adherent cell population highly enriched in AM. After the medium change, a nonspecific PDE inhibitor (IBMX, 100 μM) or vehicle was added to the cells. After a 30-min incubation, cells were stimulated with various combinations of IL-4 (5 ng/ml) or forskolin (100 μM) as described in RESULTS. After 20 h, cells were harvested, and lysates were assayed for arginase activity.

Data analysis. Data are expressed as means ± SE except for luciferase activity, which is shown as mean and range. Statistics used were paired and unpaired t-test and one-way analysis of variance with
Bonferroni test for multiple comparisons. Significance was defined as \( P < 0.05 \).

RESULTS

Treatment of RAW 264.7 cells with IL-4 or TGF-\( \beta \) for 20 h caused a 16- and 5-fold increase in arginase activity, respectively (Fig. 1), similar to previous reports (2, 5, 13, 23). The concentration of rolipram used in this study has been shown to increase cellular cAMP levels in RAW 264.7 cells by 30–40\% and to effect significant changes in cell responses (1, 8). We confirmed the effects on cAMP levels in our experiments (3.53 ± 0.10 pmol cAMP/mg protein vs. 4.57 ± 0.34 pmol cAMP/mg protein, control vs. 10 \( \mu \)M rolipram, average ± SE for \( n = 4 \), \( P < 0.05 \)). Rolipram alone at 10 \( \mu \)M induced arginase activity by only 1.5-fold. However, combining rolipram with the cytokines resulted in a marked synergistic response compared with the responses to individual stimuli. Relative to untreated controls, arginase activity in cells treated with IL-4 + rolipram or TGF-\( \beta \) + rolipram was increased by 234- and 25-fold, respectively (Fig. 1). As arginase activity represents the sum of the activities of arginases I and II and no isozyme-specific arginase inhibitors have been developed, additional studies were performed to identify the isozymes(s) responsible for the increased activity. Western blotting suggested that the increased arginase activity in cells treated with IL-4 or TGF-\( \beta \) in the presence or absence of rolipram was due primarily, if not entirely, to increased levels of arginase I (Fig. 2). As levels of arginase I in lysates from RAW 264.7 cells treated with rolipram alone were below the limit of detection by Western blot (Fig. 2), the contribution of arginase I to arginase activity under these conditions could not be evaluated.

Because of the limitations of Western blotting, real-time PCR was used to more fully evaluate the individual responses of arginases I and II, as well as the extent to which changes in arginase activity reflected changes in arginase expression at the pretranslational level. IL-4 and TGF-\( \beta \) markedly induced arginine I mRNA, a response that was further amplified by rolipram (Table 2). Arginase II mRNA was modestly induced by IL-4 or rolipram alone, and the combination of rolipram and IL-4 was only slightly greater than additive (Table 2). No change in arginase II mRNA was observed following treatment with TGF-\( \beta \) in either the absence or presence of rolipram. Together with the Western blot results, the mRNA data support the conclusion that rolipram and the cytokines synergistically increased arginase activity primarily, if not entirely, to increased levels of arginase I.

Table 2. Changes in abundance of arginase I and II mRNAs following treatment of RAW 264.7 cells with IL-4 or TGF-\( \beta \) in the absence or presence of rolipram

<table>
<thead>
<tr>
<th>Cytokine, 6 h</th>
<th>Arginase I</th>
<th>Arginase II</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>+ Rolipram</td>
<td>- Rolipram</td>
</tr>
<tr>
<td>IL-4</td>
<td>380±57</td>
<td>288±221</td>
</tr>
<tr>
<td>TGF-( \beta )</td>
<td>120±35</td>
<td>1.1±0.1</td>
</tr>
<tr>
<td>Cytokine, 20 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>IL-4</td>
<td>581±136</td>
<td>31±0.6</td>
</tr>
<tr>
<td>TGF-( \beta )</td>
<td>223±119</td>
<td>1.1±0.1</td>
</tr>
</tbody>
</table>

Fig. 1. Effect of IL-4, transforming growth factor (TGF)-\( \beta \), and rolipram on arginase activity in RAW 264.7 cells. Arginase-specific activity was determined following 20-h cytokine stimulation in the absence (open bars) or presence (filled bars) of rolipram. In all treatments arginase activity was elevated significantly above control (\( P < 0.05 \)). Within each group rolipram significantly increased arginase activity (\( P < 0.05 \)) and resulted in a marked synergism with IL-4 and TGF-\( \beta \) (\( P < 0.05 \)). Results are expressed as means ± SE for 3 independent experiments.

Fig. 2. Effect of IL-4, TGF-\( \beta \), and rolipram (R) on arginase I protein abundance in RAW 264.7 cells. Arginase I levels in lysates (20 \( \mu \)g each lane) of cells after 20 h of treatment with the indicated agents were determined by Western blotting (top). Total mouse liver extract (1 \( \mu \)g) served as a positive control for arginase I. Sypro Ruby staining of the membrane before immunoblotting is shown to demonstrate equal loading of lysate samples (bottom); staining of the mouse liver extract was too low to show up on this exposure. Results for duplicate cultures are shown and are representative of 3 independent experiments. CT, control.

Table 2. Changes in abundance of arginase I and II mRNAs following treatment of RAW 264.7 cells with IL-4 or TGF-\( \beta \) in the absence or presence of rolipram

Values are expressed as means ± SE for 3 independent experiments, each measured in duplicate. mRNA levels at 6 and 20 h were calculated by the comparative threshold method and are expressed relative to the levels in the corresponding untreated control cells. The level of HPRT mRNA was used as an internal reference for all conditions. TGF, transforming growth factor.
the conclusion that increases in arginase I are primarily responsible for overall increases in arginase activity.

To support the conclusion that the effect of rolipram reflected an effect of increased cAMP, the experiment was repeated with a concentration of 8-bromo-cAMP that elicits induction of arginase I similar to that obtained with rolipram alone (Table 3). Values of relative expression differed somewhat from those in Table 2, probably reflecting the fact that a different passage number of cells and different lots of cytokines were used in this experiment. Nonetheless, the patterns of response in Tables 2 and 3 were essentially the same; i.e., both rolipram and 8-bromo-cAMP synergized with the cytokines in induction of arginase I.

RAW 264.7 cells stably transfected with a luciferase reporter gene driven by a 4.78-kb murine arginase I promoter (7) were used to determine whether the increases in arginase I mRNA reflected increases in arginase I transcription. Previous studies in RAW 264.7 cells have demonstrated that the arginase I promoter contains elements required for responses to IL-4 (7, 26), and the present study demonstrates that it also contains elements responsive to TGF-β and cAMP. Similar to the pattern seen in the real-time RT-PCR results, rolipram alone had no effect on arginase I promoter activity but significantly enhanced induction by IL-4 and TGF-β (Fig. 3). These results demonstrated that there was a transcriptional component to induction of arginase I by these stimuli.

To determine whether the results obtained with the murine macrophage line were relevant to regulation of arginase expression in humans, similar studies were conducted with primary cultures of human AM. Owing to the limited amounts of human AM that were available for these studies, our objective was simply to ascertain whether arginase activity in human AM was inducible by IL-4 and whether the response to IL-4 could be amplified by agents that elevate intracellular cAMP levels. Because of the variation in arginase-specific activities from different individuals, arginase activities are expressed relative to the arginase activities in untreated AM for each patient. Arginase-specific activities in untreated AM ranged from 0.95 to 2.77 nmol min⁻¹ mg protein⁻¹, similar to the specific activities in unstimulated RAW 264.7 cells (Fig. 1). To test the basic hypothesis that agents that elevate intracellular cAMP levels can enhance arginase I induction by IL-4 in human AM, a combination of forskolin (an adenylate cyclase activator) and IBMX (a nonspecific PDE inhibitor) was used to maximally increase intracellular cAMP levels. Whereas there was no significant response to IL-4 alone and the combination of forskolin and IBMX increased arginase activity by only 1.8-fold, the combination of these two agents with IL-4 resulted in a fourfold increase in arginase activity (Fig. 4). The absence of a response to IL-4 alone was in contrast to results found with the murine macrophage cells. Nonetheless, these results did demonstrate that agents that raise intracellular cAMP levels can greatly enhance induction of arginase I by IL-4 in both human and murine macrophage cells.

**DISCUSSION**

It is becoming apparent that elevations in arginase accompany a variety of pathological states, including pulmonary diseases such as asthma, silicosis, pulmonary hypertension,

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**Table 3. Changes in abundance of arginase I and II mRNAs following treatment of RAW 264.7 cells with IL-4 or TGF-β in the absence or presence of 20 μM 8-bromo-cAMP**

<table>
<thead>
<tr>
<th>Cytokine, 6 h</th>
<th>Arginase I</th>
<th>Arginase II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−cAMP</td>
<td>+cAMP</td>
</tr>
<tr>
<td>None</td>
<td>1.0±0.1</td>
<td>4.8±0.6</td>
</tr>
<tr>
<td>IL-4</td>
<td>185±15</td>
<td>848±62</td>
</tr>
<tr>
<td>TGF-β</td>
<td>23±2.6</td>
<td>97±5.1</td>
</tr>
</tbody>
</table>

mRNA levels at 6 h posttreatment were determined as described in Table 2. Values are expressed as means ± SE for 2 independent experiments, each performed in duplicate.
and bleomycin-induced pulmonary fibrosis (6, 15–19, 27, 30, 38). The cytokines IL-4 and TGF-β, which are implicated in the pathogenesis of various lung diseases, also are inducers of arginase and likely contribute to the increased arginase levels seen in these diseases. Because of interest in the use of inhibitors of PDE4, the principal PDE in immune cells, in the treatment of inflammatory lung diseases such as asthma and chronic obstructive pulmonary disease (3, 11, 31, 32) and because combinations of IL-4 or TGF-β with cAMP analogs can synergistically induce arginase in multiple cell types (20, 33, 34), the use of PDE4 inhibitors in diseases involving elevated IL-4 or TGF-β levels could have effects on arginase expression that might exacerbate some pathophysiological features. We therefore tested the hypothesis that PDE4 inhibitors would amplify induction of arginase by IL-4 or TGF-β in macrophages.

Rolipram alone produced only a slight increase in arginase activity in RAW 264.7 cells, similar to the modest increases elicited by rolipram or IBMX in rabbit and rat AM (9, 10). As seen for combinations of cAMP analogs and cytokines (20), PDE4 inhibition synergized with IL-4 and TGF-β to greatly enhance arginase activity in RAW 264.7 cells. The induction by cytokines, with or without rolipram, was much greater for arginase I mRNA than for arginase II mRNA. The fact that increases in arginase activity more closely paralleled the large increases in arginase I protein abundance and mRNA than the modest changes in arginase II mRNA levels supports the conclusion that increases in arginase I are primarily responsible for the observed increases in total arginase activity. Experiments using cells transfected with a reporter construct driven by the arginase I promoter showed that arginase I expression was induced at the level of transcription. As the synergistic effects of PDE4 inhibition on arginase I induction by IL-4 and TGF-β were qualitatively similar at the levels of transcription, protein abundance, and enzyme activity, the results indicate that the predominant response was at the level of transcription, although regulation at additional posttranscription steps cannot be ruled out. The potentiating effect of rolipram on arginase I induction by cytokines may be a general feature as it also enhanced arginase I induction by IL-10 in RAW 264.7 cells but to a much lesser extent than with either IL-4 or TGF-β (data not shown).

To determine whether our observations of RAW 264.7 cells are relevant to regulation of arginase expression in humans, similar experiments were performed on human AM. Similar to the results obtained with rolipram in RAW 264.7 cells and rabbit AM (9) or with IBMX in rat AM (10), the combination of forskolin plus IBMX produced only a slight increase in arginase activity in human AM. In contrast to the induction seen in murine macrophages, however, IL-4 alone had no detectable effect on arginase activity in human AM, consistent with results reported by other investigators for human monocytes and macrophages (24, 28, 29). Although the effects of forskolin plus IBMX on arginase were mild, their combination with IL-4 greatly increased arginase activity in human AM, agreeing qualitatively with the results obtained with RAW 264.7 cells. In contrast to conclusions drawn in other studies (29), these results demonstrate that arginase is not simply incapable of being induced by IL-4 in human macrophages but that inducibility by IL-4 is highly context dependent. This may be relevant to the finding that increased numbers of arginase I-positive cells were present in BAL fluid from human asthematics (38). In our studies elevated cAMP levels were permissive for induction of arginase by IL-4 in human AM. These results emphasize the need for further studies to determine whether combinations of stimuli are a general requirement for significant induction of arginase in human AM.

The potential consequences of enhancing arginase expression in lung by PDE4 inhibitors are worth brief consideration. Both NO-dependent and NO-independent effects are possible. Because both NO synthase and arginase utilize arginine, arginase-dependent limitation of NO production can occur, as shown by effects of arginase on airway hyperresponsiveness in a guinea pig model of allergic asthma (16) and on induced airway smooth muscle relaxation (14). Elevated vascular arginase also can limit NO-dependent vasodilation (37). In addition, reduced arginine availability can result in uncoupling of NO synthase to generate superoxide (36), resulting in cell and tissue damage. Other possible consequences of increased arginase activity are increased production of polyamines, which can promote cell proliferation and airway remodeling, and increased synthesis of proline, which could support collagen synthesis in airway remodeling and pulmonary fibrosis (15).

In summary, several important new findings arose from this study: First, PDE4 inhibition greatly enhanced induction of arginase in murine macrophage cells by IL-4 and TGF-β, principally via induction of arginase I. Second, these effects occurred at the level of transcription as shown by activation of the arginase I promoter. Third, a combination of IL-4 and agents that cause increases in intracellular cAMP synergized to elicit a dramatic induction of arginase activity in human AM, qualitatively similar to the results observed for murine macrophage cells, thus demonstrating for the first time that IL-4 can play a direct role in induction of arginase in human AM. Together, our results indicate that PDE4 inhibitors or other agents that elevate intracellular levels of cAMP may further enhance the elevated expression of arginase seen in inflammatory disorders in which IL-4 or TGF-β play major roles. Therefore, arginase expression and its potential consequences should be evaluated whenever such agents are proposed for treatment of inflammatory lung diseases in which IL-4 or TGF-β predominate.

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